The α7-nicotinic Acetylcholine Receptor and MMP-2/-9 Pathway Mediate the Proangiogenic Effect of Nicotine in Human Retinal Endothelial Cells

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Purpose. Nicotine, the active component of cigarette smoke, has been found to stimulate angiogenesis in several experimental systems. In this study, the Matrigel duplex assay (Matrigel; BD Biosciences, Franklin Lakes, NJ) and the rat retinal explant assay were used to explore the molecular mechanisms underlying the proangiogenic effects of nicotine in endothelial cells.

Methods. Western blot analysis was performed to determine the nicotinic acetylcholine receptor (nAChR) subtypes expressed on primary human retinal microvascular endothelial cells (HRMECs). The angiogenic effect of nicotine in the retina was evaluated with the duplex assay. The results obtained from the assay were confirmed by the rat retinal explant angiogenesis assay. ELISAs were used to measure MMP-2, -9, and -13 levels in HRMEC culture supernatants. The role of α7-nAChRs in nicotine-induced angiogenesis was examined by siRNA techniques.

Results. Nicotine-induced angiogenesis required nAChR function and was associated with the upregulation of MMP-2 and -9 in HRMECs. Specifically, α7-nAChRs mediated the stimulatory effects of nicotine on retinal angiogenesis and MMP levels. Treatment of HRMECs with α7-nAChR antagonists ablated nicotine-induced angiogenesis. The inhibitory actions of α7-nAChR antagonists correlated with the suppression of MMP-2 and -9 levels in HRMECs.

Conclusions. The α7-nAChR is vital for the proangiogenic activity of nicotine. The α7-nAChRs expressed on HRMECs upregulate levels of MMP-2 and -9, which stimulate retinal angiogenesis. The data also suggest that α7-nAChR antagonists could be useful agents for the therapy of angiogenesis-related retinal diseases. (*Invest Ophthalmol Vis Sci.* 2011;52:4428–4438) DOI:10.1167/iovs.10-5461

Neovascular diseases of the retina, such as diabetic retinopathy (DR) and age-related macular degeneration (ARMD), constitute the leading cause of blindness in developed countries.[1,2] These proliferative retinopathies involve the pathologic growth of new blood vessels as a result of hypoxic stimuli such as ischemia or inflammation.[3] Laser photocoagulation, the existing therapy for retinopathies, can destroy postmitotic retinal neurons and permanently affect visual function. Therefore, pharmacologic agents that possess antiangiogenic activity without destroying retinal tissue could lead to new treatments for this constellation of retinal diseases.[3–5]

Cigarette smoking is regarded as a modifiable risk factor for diabetic retinopathy.[1,6] The relationship between smoking and diabetic retinopathy is complex and less well understood; however, several reports suggest that smoking is associated with the incidence and progression of diabetic retinopathy.[7–14] Data reported by Muhlhauser et al.[15] showed that smoking doubles the risk of proliferative retinopathy and promotes the progression from background to proliferative retinopathy in type 1 diabetes. In contrast, studies by Moss et al.[16,17] did not show a significant correlation of smoking with the risk of diabetic retinopathy. It has been suggested that the failure to correlate diabetic retinopathy with cigarette smoking may be due to increased mortality in smokers.[18,19] However, in a recent paper, Klein et al.[7] showed that smoking is clearly involved in the 25-year cumulative incidence of visual impairment in type 1 diabetes. The data are in agreement with studies that have listed smoking as a modifiable risk factor in diabetic retinopathy.[1,6] Smoking worsens other problems, such as large-vessel disease and renal failure, and these changes in turn can exacerbate retinopathy.[20–22] Taken together, there is a growing body of evidence to suggest that smoking is involved in the pathophysiology of diabetic retinopathy. Although cigarette smoke is a complex mixture of more than 4000 compounds, nicotine is the active and addictive component.[23] Several studies have shown that nicotine promotes angiogenesis in experimental models of cancer, atherosclerosis, and retinal neovascularization.[23–30] In addition, nicotine stimulated angiogenic tube formation in vitro by both retinal and choroidal endothelial cells.[25] Furthermore, the administration of nicotine enhanced the size and severity of choroidal neovascularization (CNV) in C57BL6 mice.[24,25]

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The proangiogenic activity of nicotine is mediated by nicotinic acetylcholine receptors (nAChRs) on choroidal and retinal endothelial cells.[23] Real-time PCR analysis showed that both choroidal and retinal endothelial cells express mRNA for α3, α5, α7, α9, β1, β3, and β4, whereas retinal endothelial cells also express α1, α6, α10, and β2.[25] However, the specific mechanism for nicotine's action in the retina has not been extensively studied. Hou et al.[31] used the laser CNV model in mice to demonstrate that nicotine-induced angiogenesis in the eye is associated with increased recruitment of bone marrow–derived progenitor cells into the newly formed vasculature in the eye. The proangiogenic effects of nicotine correlated with increased levels of retinal phospholipase A2 in vitro.[32] In cultured choroidal vascular smooth muscle cells, nicotine promotes platelet-derived growth factor (PDGF)–induced expression of matrix metalloproteinases (MMPs) and prevents vascular endothelial growth factor (VEGF)–mediated inhibition of MMP-2.[24] These studies suggest that nicotine-induced ocular angiogenesis is mediated by the transmigration and invasion of retinal (and choroidal) endothelial cells.

The administration of generalized nAChR antagonists, like hexamethonium and mecamylamine, ablated nicotine-induced CNV in mice models, suggesting that these agents can be useful in the treatment of proliferative retinopathies.[24,25] However, the disadvantage of generalized nAChR inhibitors is that they bind to all nAChR-subtypes and may display unwanted pleiotropic effects. Such considerations clearly emphasize the need for a second generation of subunit-specific nAChR inhibitors with improved specificity and antiangiogenic activity.

The α7-nAChR has been implicated in the proangiogenic activity of nicotine in atherosclerosis and cancer.[23,33] However, it is not yet known whether α7-nAChRs mediate the angiogenic effects of nicotine in retinal endothelial cells. In the present study, we showed that nicotine (at concentrations present in the plasma of an average smoker, 10^{-8}M–10^{-6}M) promotes angiogenesis in primary human microvascular retinal endothelial cells (HRMECs).[23] The proangiogenic effects of nicotine are mediated by α7-nAChRs and involve the MMP-2 and -9 pathway.[14–17] Finally, we demonstrate that α7-nAChR antagonists suppress nicotine-induced retinal angiogenesis. The development of novel α7-nAChR antagonists would not only shed light on nAChR-signaling mechanisms in retinal endothelial cells, but would also foster the hope of novel therapies for neovascular retinal disease.

Materials and Methods

Cell Culture

HRMECs (Cell Systems, Kirkland, WA) were maintained in endothelial basal medium (EBM)-2 containing growth factors and supplemented with 5% FBS (hereafter denoted EGM-2), prepared according to the manufacturer's protocol (Lonza Technologies, Basel, Switzerland). All experiments using HRMECs were performed between passages 3 and 8. During the duplex assay, HRMECs were serum starved in reduced EGM-2 (referred to hereafter as EGM-R), which was composed of EBM-2 containing one fourth the concentration of growth factors and 0.5% FBS.

The generalized MMP inhibitor GM6001, specific inhibitors for MMP-2 and -9, and the dual MMP-2/9 inhibitor were obtained from Calbiochem (Merck-EMD Biosciences, Darmstadt, Germany).

Matrigel Duplex Assay

The Matrigel duplex assay (Matrigel; BD Biosciences, Franklin Lakes, NJ) that we used is a modification of an assay described by Browning et al.[34] HRMECs were serum starved in EGM-R for 24 hours. Subsequently, the cells were harvested and resuspended in 2× EGM-R (containing EBM-2 with a one-half volume of growth factors and 1% FBS) at a concentration of 1.6×10^7 cells/mL. The cell suspension was then treated with the test compounds, diluted 1:1 in growth factor–reduced Matrigel (GFR-Matrigel). A 6-μL aliquot of this mixture was pipetted as a drop onto the center of each well of an eight-well chamber slide (Fig. 1A). These spots of cells mixed with GFR-Matrigel were allowed to polymerize at 37°C for 1 hour, after which they were bathed in 200 μL EGM-R medium and incubated for 24 hours at 37°C. At this stage, the HRMECs associated and formed a complex network of angiogenic tubules within and up to the edge of the GFR-Matrigel spot. The medium was then gently aspirated, and the border of the GFR-Matrigel spot was outlined on the bottom surface of the plate with a fine-tipped marker. This black line was examined under a microscope to confirm that it accurately traced the periphery of the GFR-Matrigel spot. A second GFR-Matrigel solution was prepared by diluting GFR-Matrigel with EGM-R (1:1) along with the test compounds. Two hundred microliters of this diluted solution was pipetted over the drops and incubated for 1 hour at 37°C (Fig. 1B). The cells were then bathed in 300 μL EGM-R and incubated for 24 hours at 37°C. Subsequently, each spot was observed by phase-contrast microscopy (DMIL LED; Leica, Bannockburn, IL). Under the phase-contrast microscope, the black line demarcating the two layers appeared as a dark band because of the three-dimensional nature of the culture (Fig. 1C). Three inde-

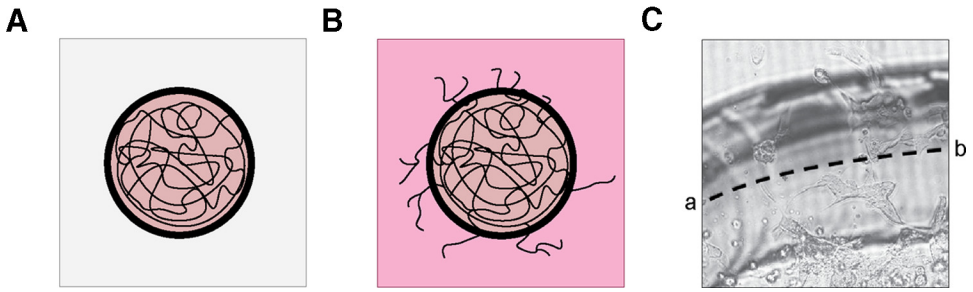

Figure 1. The Matrigel duplex assay. (**A**) The first layer was composed of HRMECs mixed with Matrigel (*light pink*) synthetic matrix. The HRMECs differentiated to form angiogenic tubules extending up to the boundary of this first layer. The boundary of the first layer was outlined with a black marker before the second layer was added to serve as a visual guide of the interface, indicated by the *black circle*. (**B**) After 24 hours, a second layer was added on top of the first spot. This addition resulted in the growth and invasion of preexisting retinal angiogenic sprouts into the secondary layer (*dark pink*). (**C**) After the HRMEC tubules migrated into the second layer, the chamber slides were observed by phase-contrast microscopy. Because of the 3-D nature of the culture, the drawn *black line* appeared as a narrow band. On the photographed image, a *dotted line* (marked a–b) was drawn in the middle of this black band. Line a–b was taken as the interface between the two layers. All angiogenic tubules that cross line a–b are counted in a randomized double blind fashion by three independent observers.

pendent fields of these duplex cultures were photographed by phase-contrast microscopy (DMIL LED; Leica). On the photographed image of these cultures, a dotted line was drawn in the middle of the dark band to mark the interface between the primary and secondary GFR-Matrigel layers (Fig. 1C, dotted line a–b). The number of angiogenic sprouting vessels that crossed line a–b was counted in three randomized independent fields in a double-blind fashion. Each data point was calculated in duplicate, and the whole experiment was repeated twice.[34,35]

Lysates and Western Blot Analysis

HRMEC lysates were made using the NP-based lysis protocol.[36,37] The relative expression of the indicated proteins was analyzed by Western blot. Polyclonal α3-, α4- and α7-nAChR antibodies (Abcam, Cambridge, MA), β2- and β3-nAChR antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA), and GAPDH antibody (Trevigen, Inc., Gaithersburg, MD) were used for the immunoblot experiments.[37,38] The results of the Western blot assays were quantitated by densitometry (Gel Documentation System, with Quantity One 4.5.2 analysis software; Bio-Rad, Hercules, CA).

siRNA Transfection and Assays

Chemically synthesized, double-stranded siRNA for α3- and α7-nAChRs were purchased from Ambion Biotechnology (Austin, TX). The transfection experiments were performed in HRMECs during passages 3 to 8. Asynchronous HRMECs were harvested and replated in six-well plates at approximately 40% confluence in EGM containing 5% FBS in the absence of antibiotics, at a density of 250,000 cells/well. Each well was transfected with 50 nM siRNA.[37,38] An aliquot of 25 μL of transfection reagent (Oligofectamine; Invitrogen Corp. Carlsbad, CA) was used per transfection. The siRNA was diluted to a final volume of 300 μL in reduced-serum medium (OPTI-MEM; Invitrogen) and incubated for 5 minutes at room temperature. Similarly, the transfection reagent was diluted to a total volume of 200 μL and incubated for 5 minutes at room temperature. Subsequently, the siRNA solution was added to the reagent, and the mixture was incubated at room temperature for 45 minutes. Meanwhile, the cells were washed once in PBS and once in the reduced-serum medium, and the transfection reagent–siRNA complexes were added to the cells. The cells were incubated at 37°C for 6 hours. After 6 hours, the siRNA complexes were aspirated and EGM containing 7.5% FBS was added to the cells. The cells were then incubated for 18 hours at 37°C. Eighteen hours after transfection, the cells were rendered quiescent for 24 hours by incubation in EGM-R, after which the Matrigel duplex assay was performed. A nontargeting siRNA sequence (Ambion) was used as a control siRNA (at a concentration of 50 nM) for the transfection experiments.[37,38] Each transfection was performed in duplicate, and the entire assay was repeated twice.

Western blot experiments were performed to assess the expression of proteins after siRNA transfection in HRMECs.[37,38] The results of the Western blot assays were quantitated by densitometry (Gel Documentation System, with Quantity One 4.5.2 analysis software; Bio-Rad).

Rat Retinal Explant Assay

All animal procedures used were in agreement with the NIH Guide for the Care and Use of Laboratory Animals, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the Joan C. Edwards School of Medicine, Marshall University.

Twenty-week-old male Zucker lean rats (Charles River Laboratories, Wilmington, MA) were used in the studies and were fed ad libitum (5008 diet; Purina, Indianapolis, IN). The rats were anesthetized with a ketamine-xylazine cocktail. Once full anesthesia was reached, the animals were killed via decapitation, and the retinas were isolated.[5,39] Meanwhile, 48-well tissue culture plates were coated with 200 μL of GFR-Matrigel and polymerized for 1 hour at 37°C. The explants were placed on the GFR-Matrigel–coated wells, covered with an additional 800 μL of the matrix, and allowed to polymerize for 1 hour at 37°C. Subsequently, EGM-R containing the test compounds was added on top of the Matrigel. The medium and test compounds were added to the EGM-R every alternate day for 1 week. The sprouting angiogenic tubules were photographed on day 7 by phase-contrast microscope (DMIL LED; Leica). Each sample was examined in duplicate, and the whole experiment was repeated twice.

Measurement of MMPs and TIMPs in Culture Supernatants

HRMECs (passages 3–8) were plated in six-well plates at a density of 2.0×10^5 cells/well in EGM-2. After overnight incubation at 37°C, the cells were incubated in EGM-R for 24 hours. Subsequently, the medium was aspirated, and the cells were incubated with the test compounds in serum-free, growth-factor–free EBM for 24 hours. The supernatant was collected and centrifuged at 800*g* at 4°C, to remove all particles. Next, the supernatant was collected, aliquotted, snap frozen, and stored in −80°C until further analysis. ELISA kits (Novagen-EMD Biosciences) were used to analyze the levels of MMP-2, -9, and -13 and TIMP-1 and -2. The samples were diluted fivefold for MMP-2 and -9 analyses and were undiluted for MMP-13, TIMP-1, and TIMP-2 analyses.

Statistical Analysis

All data are expressed as the mean ± SEM (Prism 5; GraphPad, San Diego, CA). An analysis of variance (ANOVA) followed by a Newman-Keuls post hoc test was used to determine significant differences. All analyses were completed with a 95% confidence interval. Data were considered significant at $P < 0.05$.

Results

Nicotine Increases the Number of Invading Angiogenic Tubules in a Duplex Assay

The proangiogenic effects of nicotine were examined in HRMECs by using the Matrigel duplex assay. Quiescent HRMECs displayed very few migrating angiogenic tubules into the secondary layer (Fig. 2A, top left). Conversely, nicotine induced a dose-dependent increase in the number of migrating angiogenic tubules (Fig. 2A, top right and bottom) across the interface. The number of angiogenic tubules crossing the interface was quantitated as described above. The maximum number of migrating tubules was observed at 100 nM nicotine and remained constant thereafter (Fig. 2B). Thus, a concentration of 100 nM nicotine was used for all subsequent experiments. The concentration of nicotine in the plasma of an average smoker ranges between 10 nM and 1 μM; therefore, the concentration of nicotine used in our experiments lies within the range found in smokers.[27,28,40]

Western blot analysis showed that α3, α7, β2, and β3 nAChRs were expressed on the HRMECs (Fig. 2C). HEK293 cells were used as the negative control for the experiment, and A549 human non–small-cell lung cancer cells were used as the positive control.[38]

Proangiogenic Activity of Nicotine Is Mediated by nAChRs on HRMECs

The next series of experiments addressed whether the proangiogenic effect of nicotine in HRMECs requires nAChR function. Matrigel duplex assays showed that treatment of HRMECs with 100 nM nicotine caused a significant increase ($P < 0.05$) in the number of migrating angiogenic tubules over that in the control (Fig. 3A). The presence of 1 μM of the generalized nAChR antagonist mecamylamine (MCA) abrogated the proangiogenic activity of nicotine.[37,38] However, the treat-

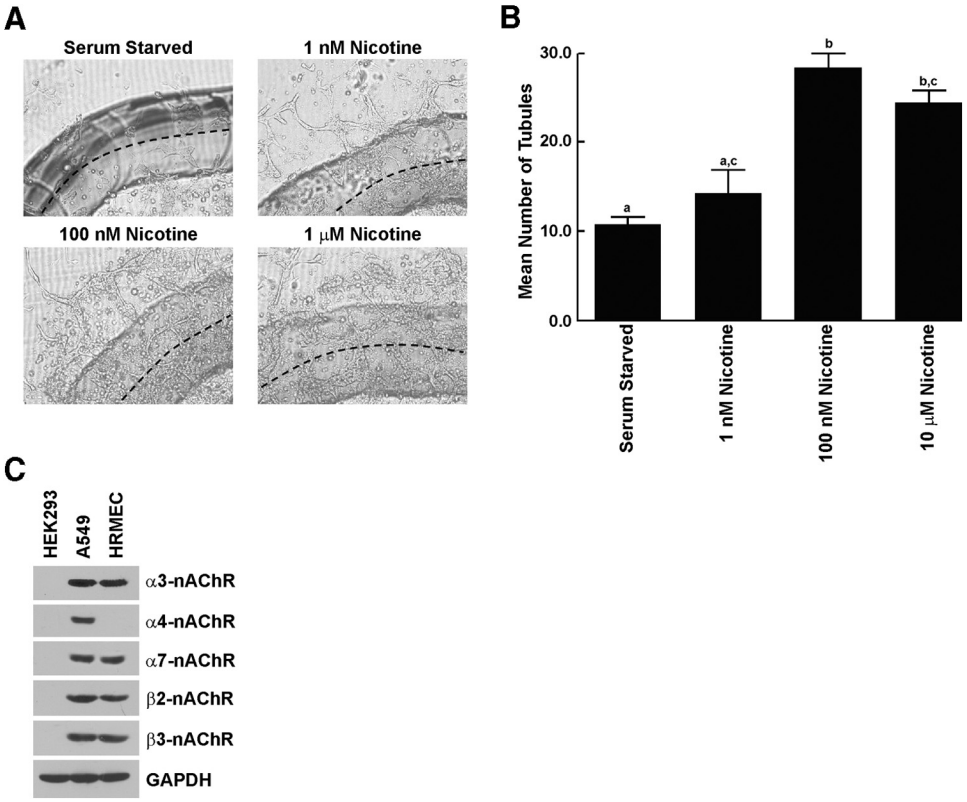

FIGURE 2. Nicotine stimulated retinal angiogenesis in a dose-dependent manner. (**A**) Matrigel duplex assays indicated that nicotine promoted the migration of retinal angiogenic tubules into the second layer. Only a few angiogenic tubules migrated across the *dotted line* interface in quiescent HRMECs (*top left*). The treatment of HRMECs with various doses of nicotine caused the invasion of retinal tubules into the secondary Matrigel layer, with the maximum effect observed at 100 nM and 1 μM (*top right* and *bottom*). (**B**) Quantitation of the number of HRMEC tubules migrating across the *dotted interface line* demonstrated that nicotine enhanced retinal angiogenesis in a dose-dependent manner, as measured by the duplex assay. The maximum angiogenic activity of nicotine was observed at 100 nM, and this concentration was used for all further experiments. Values indicated by the same letter are not statistically significant. (**C**) HRMECs expressed multiple nAChR subunits. The human non–small-cell lung cancer cell line A549 was used as the positive control for the assay, whereas HEK293 cells were used as the negative control.

ment of HRMECs with 1 μM atropine, an antagonist of the closely related muscarinic AChR, along with 100 nM nicotine did not affect nicotine-induced angiogenic tubule formation (Fig. 3A). Taken together, these data suggest that the nicotine-induced retinal angiogenesis is mediated through nAChRs on HRMECs.

The results of these experiments were verified with a rat retinal explant assay. Rat retinal explants cultured in GFR-Matrigel displayed very little sprouting angiogenesis (Fig. 3B, top left). However, stimulation of these explants with 100 nM nicotine caused robust angiogenic sprouting (Fig. 3B, top middle). The proangiogenic activity of nicotine was ablated by 1 μM MCA (Fig. 3B, top right), whereas it was unaffected by atropine (Fig. 3B, bottom left). VEGF (100 ng/mL) was used as a positive control for the assay (Fig. 3B, bottom right).

Nicotine-Induced Angiogenesis Is Mediated by MMP-2 and -9 in HRMECs

Next, attempts were made to elucidate the molecular mechanism by which nicotine enhanced angiogenesis in the Matrigel duplex and rat retinal explant assays. The upregulation of MMP levels is one of the major mechanisms contributing to retinal angiogenesis[41–44]; therefore, we conjectured that nicotine-induced angiogenesis could be due to increased expression of MMPs. The generalized MMP inhibitor GM6001 (20 μM) was found to ablate nicotine-induced angiogenesis (Fig. 4A), suggesting that the proangiogenic effects of nicotine were mediated by the MMP pathway.

The role of specific MMPs in nicotine-induced angiogenesis was examined by using chemical inhibitors of specific MMP proteins. The gelatinases MMP-2 and -9 have been shown to play a vital role in angiogenesis-related diseases, such as diabetic retinopathy and ARMD.[41,45–50] Clinical studies in diabetic patients, as well as in animal models of diabetic retinopathy, show increased levels of MMP-2 and -9 in their plasma.[51–53] The elevation of MMP-2 and -9 in diabetic retinopathy is believed to contribute to disruption of tight junctions, stimulation of endothelial cell migration and increase in vascular permeability.[54] In addition, growth factors such as bFGF and VEGF upregulate the secretion of MMP-2 and -9 in retinal cells during angiogenesis.[55] Therefore, we conjectured that perhaps nicotine behaves in a manner analogous to growth factors and promotes the secretion of MMP-2 and 9 from HRMECs. We found that 20 μM of an inhibitor that blocked both MMP-2 and -9 (referred to as MMP-2/-9 inhibitor in Fig. 4A) reversed nicotine-induced angiogenesis in duplex assays. This result indicates that MMP-2 and/or -9 contribute to the proangiogenic effects of nicotine in HRMECs. The next series of duplex assays were completed to identify which of the MMPs (-2, -9, or both) contribute to nicotine-induced angiogenesis.

HRMECs were rendered quiescent for 24 hours, after which they were treated with 100 nM of nicotine in the presence of 20 μM MMP-2 inhibitor, 20 μM MMP-9 inhibitor, or a combination of both inhibitors (referred to as MMP-2 + 9; Fig. 4B). Subsequently, a duplex assay was performed, and the number of angiogenic sprouts migrating across the interface was measured. Treatment of HRMECs with 20 μM of the MMP-2 inhibitor or 20 μM of the MMP-9 inhibitor individually did not affect nicotine-induced angiogenesis; however, when both inhibitors were added in combination, the angiostimulatory effect of nicotine was ablated (Fig. 4B). The results of these experiments were verified using the rat retinal explant assay, which yielded similar data (Fig. 4C). Results from both retinal assays suggest that the proangiogenic activity is, in part, due to nicotine-induced secretion of MMP-2 and -9 from HRMECs.

Nicotine Stimulates the Production of MMP-2 and -9 via nAChRs

ELISAs demonstrated that nicotine potently induced levels of MMP-2 and -9 in HRMEC culture supernatants (Fig. 5A). Since the MMP-9 inhibitor used in the previous set of results is also

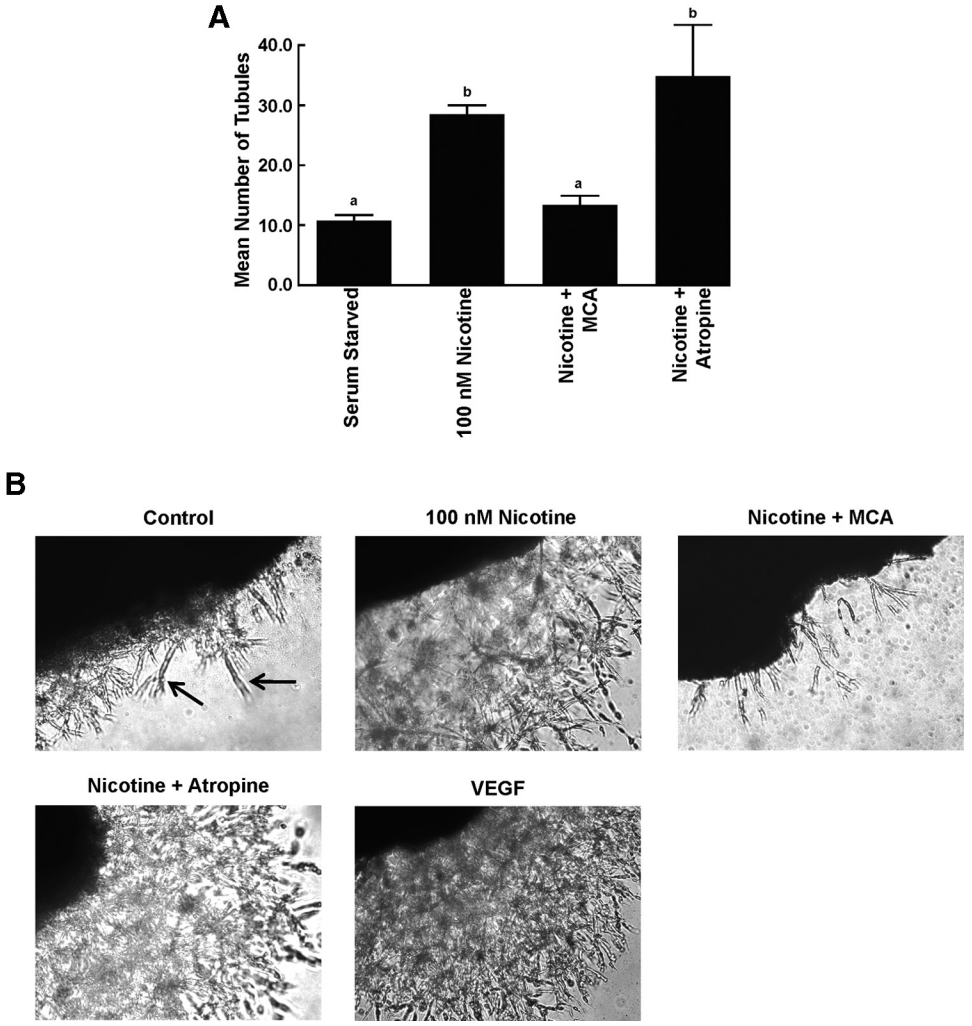

FIGURE 3. The proangiogenic effect of nicotine was mediated by nAChRs on HRMECs. (**A**) Matrigel duplex assays showed that the proangiogenic effect of 100 nM nicotine was suppressed by 1 μM of the generalized nAChR antagonist mecamylamine (referred to as MCA), whereas treatment with 1 μM of the closely related muscarinic receptor antagonist atropine had no effect. Values indicated by the same letter are not statistically significant. (**B**) The results were verified by a rat retinal explant assay. Rat retinal explants were seeded on GFR-Matrigel in the presence or absence of the indicated test compounds. The control explants (*top left*) displayed very little angiogenic sprouting (*arrows*). The treatment of explants with 100 nM nicotine (*top middle*) induced robust angiogenic sprouting. When the nicotine treatment was performed in the presence of 1 μM MCA, the number of angiogenic tubules were markedly reduced (*top right*). However, 1 μM atropine had no effect on nicotine-induced sprouting angiogenesis (*bottom left*). VEGF (100ng/mL) was taken as the positive control for the assay (*bottom right*).

cross-reactive with MMP-13, we analyzed whether nicotine affects MMP-13 levels. Nicotine did not have any effect on the MMP-13 levels in HRMECs (Fig. 5A). These observations suggest that nicotine promotes angiogenesis in HRMECs via the MMP-2/-9 pathway.

Next, we examined whether nicotine stimulates the production of MMP-2 and -9 via nAChRs. MMP ELISAs demonstrated that nicotine-induced MMP-2 and -9 secretions were abrogated by 1 μM of the generalized nAChR antagonist MCA (Fig. 5A), whereas they were unaffected by 1 μM generalized muscarinic AChR antagonist atropine. Taken together, our data illustrate that nicotine stimulates retinal angiogenesis by promoting the secretion of MMP-2 and -9 in an nAChR-dependent manner.

The migratory activity of MMPs is counterbalanced by the tissue inhibitor of metalloproteinases (TIMP) family of proteins in the retina.[43,56,57] The effect of nicotine on TIMP-1 and -2 (which bind to MMP-2 and -9) levels was investigated. Nicotine suppressed the levels of both TIMP-1 and -2 in HRMECs (Fig. 5B). The TIMP-1 and -2 levels in untreated HRMECs were 0.38 $\pm$ 0.02 and 36.25 $\pm$ 2.05 ng/mL, respectively. Treatment with 100 nM nicotine lowered both TIMP-1 (0.15 $\pm$ 0.001 ng/mL) and TIMP-2 (7.52 $\pm$ 0.30 ng/mL) levels. The TIMP levels in untreated cells were taken as 100%, and the effect of nicotine on these levels are graphically represented as the percentage of control (Fig. 5B).[58,59] Our data suggest that nicotine promotes the onset of retinal angiogenesis by upregulating promigratory proteins such as MMP-2 and -9, with concomitant downregulation of their antimigratory binding partners, TIMP-1 and -2.

Nicotine-Induced Retinal Angiogenesis Requires α7-nAChR Function

Previous studies have shown that the proangiogenic effects of nicotine in cancer and atherosclerosis were mediated by the α7-nAChR subunit on endothelial cells.[26–28,59,60] The α7-nAChRs have also been implicated for the proliferative and proinvasive effects of nicotine in human lung cancer cells.[33] The role of α7-nAChRs in nicotine-induced retinal angiogenesis was analyzed by siRNA methodology.[37] The depletion of α7-nAChR expression by siRNA resulted in significant ablation of the proangiogenic activity of nicotine ($P < 0.05$), whereas transfection of a nontargeting control siRNA had no effect (Fig. 6A). Most interestingly, the transfection of a closely related nAChR-subunit-siRNA, α3-nAChR-siRNA, did not have any effect on nicotine-induced angiogenesis (Fig. 6A). These experiments suggest that the α7-nAChR subunit contributes to the angiostimulatory activity of nicotine in HRMECs. Western blot analysis was performed to confirm the suppression of α7-nAChR and α3-nAChR expression on siRNA transfection (Fig. 6B).[37]

An important question that emerged was whether nicotine-induced secretion of MMP-2 and -9 was mediated by α7-nAChRs. The α7-nAChR-siRNA was used to examine this possibility. MMP ELISAs showed that nicotine-stimulated secretion of MMP-2 and -9 was reversed by α7-nAChR-siRNA transfection,

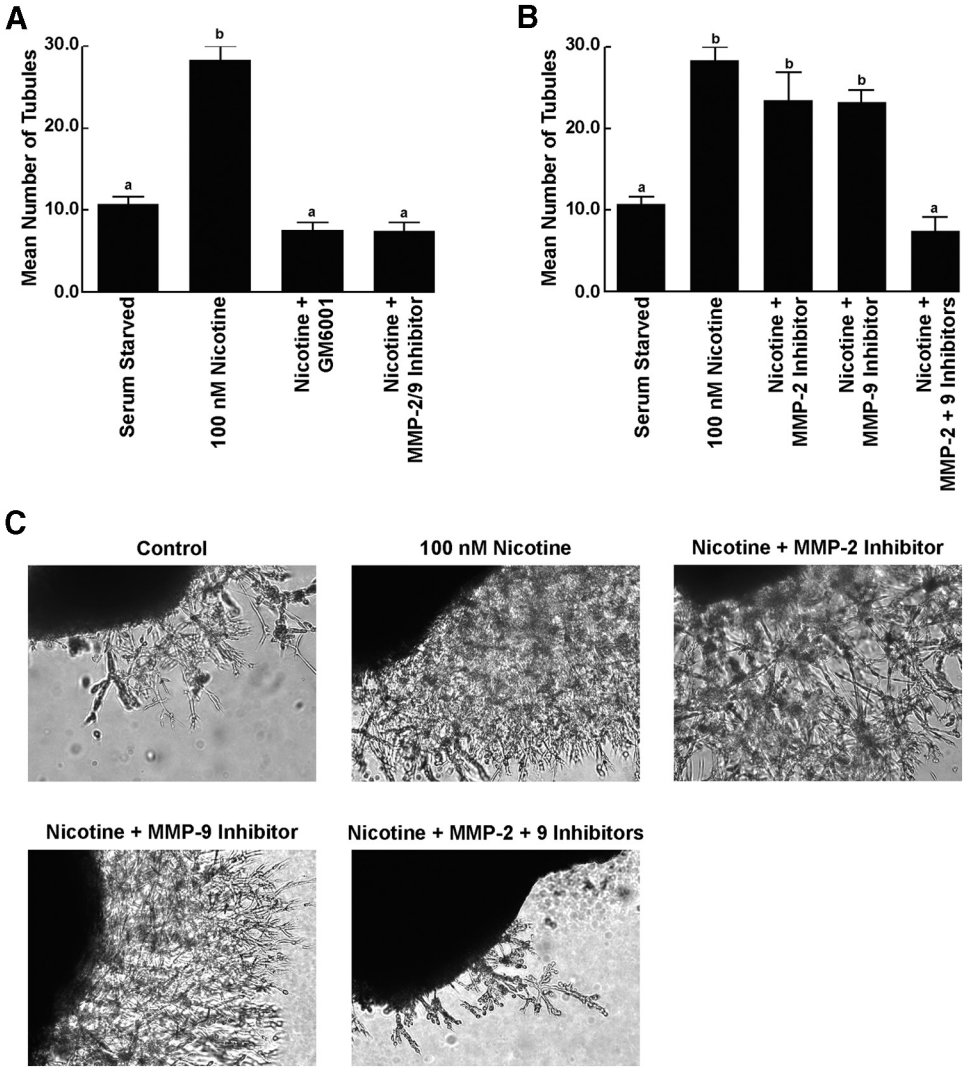

FIGURE 4. Nicotine-induced retinal angiogenesis was mediated by matrix metalloproteinases (MMPs). (**A**) Matrigel duplex assays showed that nicotine-induced angiogenesis in HRMECs was abrogated by treatment with 20 μM of the generalized MMP inhibitor GM6001. Specifically, the treatment of HRMECs with nicotine in the presence of 20 μM of the dual-specific MMP-2/9 inhibitor suppressed the proangiogenic effects of nicotine. (**B**) Nicotine-induced retinal angiogenesis was mediated by both MMP-2 and -9. The treatment of HRMECs with 20 μM of an MMP-2-specific inhibitor did not ablate nicotine-induced angiogenesis. Similar results were obtained when 20 μM of only the MMP-9 inhibitor was used. However, when HRMECs were treated with a combination of 20 μM of both MMP-2 and -9 inhibitors, nicotine-induced angiogenesis was significantly decreased ($P < 0.05$). Values indicated by the same alphabet are not statistically significant. (**C**) The results obtained were in the rat retinal explant assay.

whereas an siRNA to the closely related α3-nAChR or a control siRNA did not display any effect (Fig. 6C). Nicotine did not affect the secretion of MMP-13 in HRMECs. Taken together, our data indicate that nicotine-induced retinal angiogenesis occurs via upregulation of MMP-2 and -9, mediated specifically by α7-nAChRs.

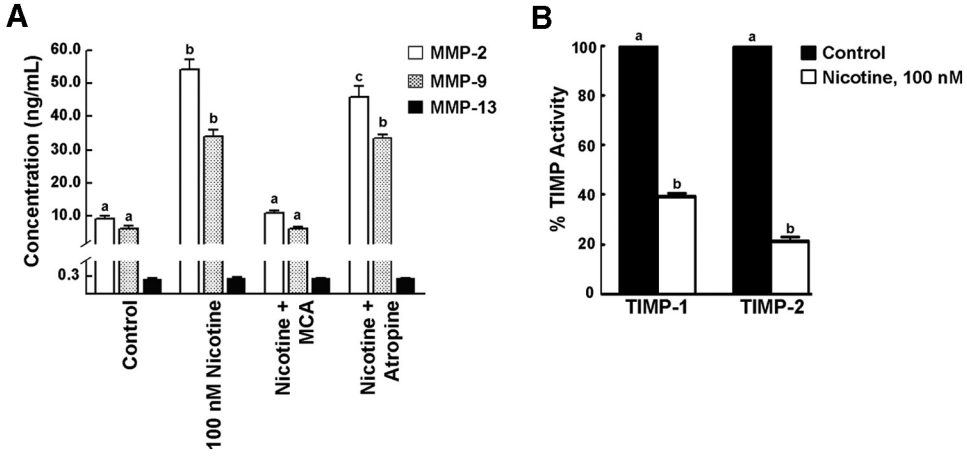

FIGURE 5. Nicotine induced the secretion of MMPs and suppressed the levels of TIMPs in HRMECs. (**A**) HRMECs were incubated with the indicated test compounds, and MMP levels were measured in culture supernatants by ELISA assays. Treatment of HRMECs with 100 nM nicotine potently stimulated MMP-2 and -9 levels in HRMEC culture supernatants. Because the MMP-9 inhibitor used in Figure 4 is also cross-reactive with MMP-13, the effect of nicotine on MMP-13 secretion from HRMECs was examined. Nicotine did not affect MMP-13 levels in HRMECs. Nicotine-induced MMP-2 and -9 secretion was suppressed by 1 μM of the generalized nAChR antagonist MCA, whereas it was unaffected by 1 μM of the muscarinic receptor antagonist atropine. (**B**) ELISA assays showed that nicotine downregulated the levels of TIMP-1 and 2 in HRMEC culture supernatants. The levels of TIMP-1 and 2 in the untreated HRMECs were taken as 100%, and the effect of nicotine on the expression of TIMP-1 and 2 was graphed as percentage of untreated controls. Values indicated by the same letter are not statistically significant.

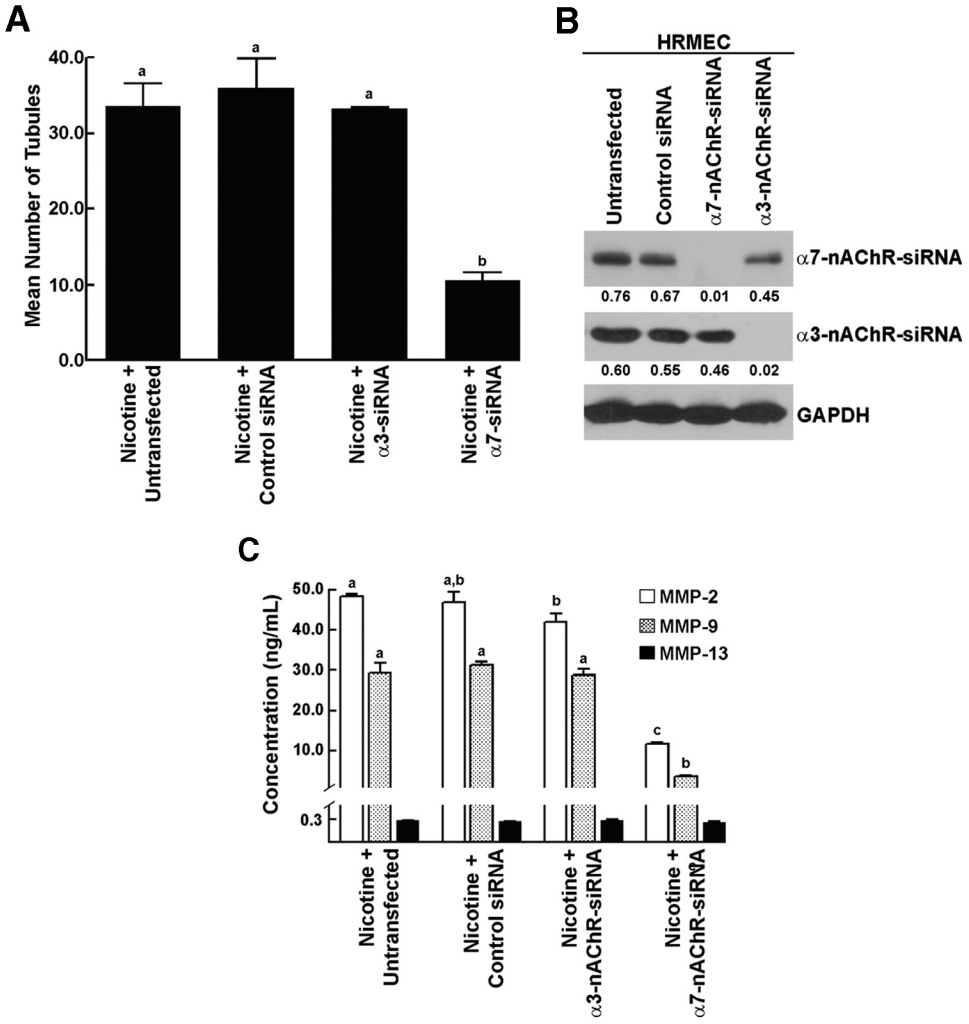

FIGURE 6. The α7-nAChR mediated the proangiogenic effect of nicotine in HRMECs. (**A**) Matrigel duplex assays indicated that the transfection of α7-nAChR-siRNA significantly attenuated nicotine-induced angiogenesis, whereas a nontargeting control-siRNA did not have any effect on the proangiogenic effects of nicotine. Most interestingly, siRNA corresponding to the closely related α3-nAChR had no influence on nicotine-induced angiogenesis, as measured by the Matrigel duplex assay. (**B**) Western blot analysis showed that both α7-nAChR and α3-nAChR levels were suppressed on siRNA transfection (*top* and *middle*). Most importantly, the transfection of α7-nAChR-siRNA had no effect on the expression of α3-nAChR (*top panel*, *lane 4*) in HRMECs and vice versa (*middle*, *lane 3*). GAPDH was used as the loading control for the Western blot experiments, and the results were quantitated by densitometric analysis. (**C**) ELISA demonstrated that suppression of α7-nAChR levels by siRNA methodology ablated nicotine-induced secretion of MMP-2 and -9. However, the transfection of α3-nAChR-siRNA and nontargeting control-siRNA had no effect on nicotine-induced MMP-2 and -9 levels. MMP-13 levels were unaffected by nicotine. Values indicated by the same letter are not statistically significant.

Antagonists to α7-nAChRs Block the Proangiogenic Effects of Nicotine

Our data show that nicotine, the active component of cigarettes, can enhance retinal angiogenesis via α7-nAChRs. As a next step, we tested whether α7-nAChR antagonists could abrogate nicotine-induced angiogenesis and suppress MMP production in response to nicotine treatment. The α7-nAChR antagonists, α-bungarotoxin (α-BT), α-cobratoxin (α-CT), and methyllycaconitine (MLA), potently suppressed nicotine-induced angiogenesis in Matrigel duplex assays (Fig. 7A). Similarly, we observed that treatment of the retinal explants with α7-nAChR inhibitors ablated nicotine-induced sprouting angiogenesis (Fig. 7B, bottom).

Data obtained from the MMP Matrigel duplex assay, rat retinal explant assay, and ELISAs suggest that the proangiogenic effects of nicotine are mediated by the upregulation of MMP-2 and -9 secretion from HRMECs (Figs. 4, 5). Therefore, we wanted to assess whether α7-nAChR antagonists suppressed nicotine-induced angiogenesis via suppression of the MMP-2 and -9 levels (Fig. 7C). The antiangiogenic effects of these α7-nAChR antagonists correlated with robust inhibition of MMP-2 and -9 levels, as measured by ELISAs, whereas no effect of α7-nAChR antagonist was observed on MMP-13 levels (Fig. 7C). Given the established correlation between cigarette smoking and retinal neovascular disease,[23] α7-nAChR antagonists that block nicotine-induced angiogenesis could form the basis for novel treatment approaches for neovascular retinal diseases.

DISCUSSION

The biological activity of nicotine is mediated by high-affinity nAChRs on target cells. Traditionally associated with neuronal cells, these nAChRs have been found in non-neuronal cells such as lung cancer cells and in multiple types of cells in the eye, including retinal endothelial, choroidal endothelial, and vascular smooth muscle cells.[23,26,61] That nAChRs have been detected on non-neuronal cells emphasizes that they have functions well beyond neurotransmission. Recent studies have shown that nicotine, the active component of cigarette smoke, can stimulate angiogenesis in cancer and atherosclerosis via nAChRs expressed on endothelial cells.[27,28,62] Experimental models used to establish the angiogenic activity of nicotine in the aforementioned studies include collagen gels, Matrigel assays, and hind limb ischemia mice models.[25,27–29,62] The drawback of these models is that they are not representative of the angiogenic processes in the retina, which is characterized by the formation of new blood vessels from existing circulation. These new blood vessels grow and migrate into the inner limiting membrane and the vitreous, both of which contribute to vision loss.[3] Stitt et al.[35] and Browning et al.[34] developed a novel angiogenesis assay, the Matrigel duplex assay, which represents retinal angiogenesis in vivo. The growth and migration of new retinal vessels from preexisting vasculature is well replicated by the Matrigel duplex assay, in which preexisting retinal angiogenic tubules grow and migrate into the secondary Matrigel layer. Furthermore, these retinal endothelial sprouts

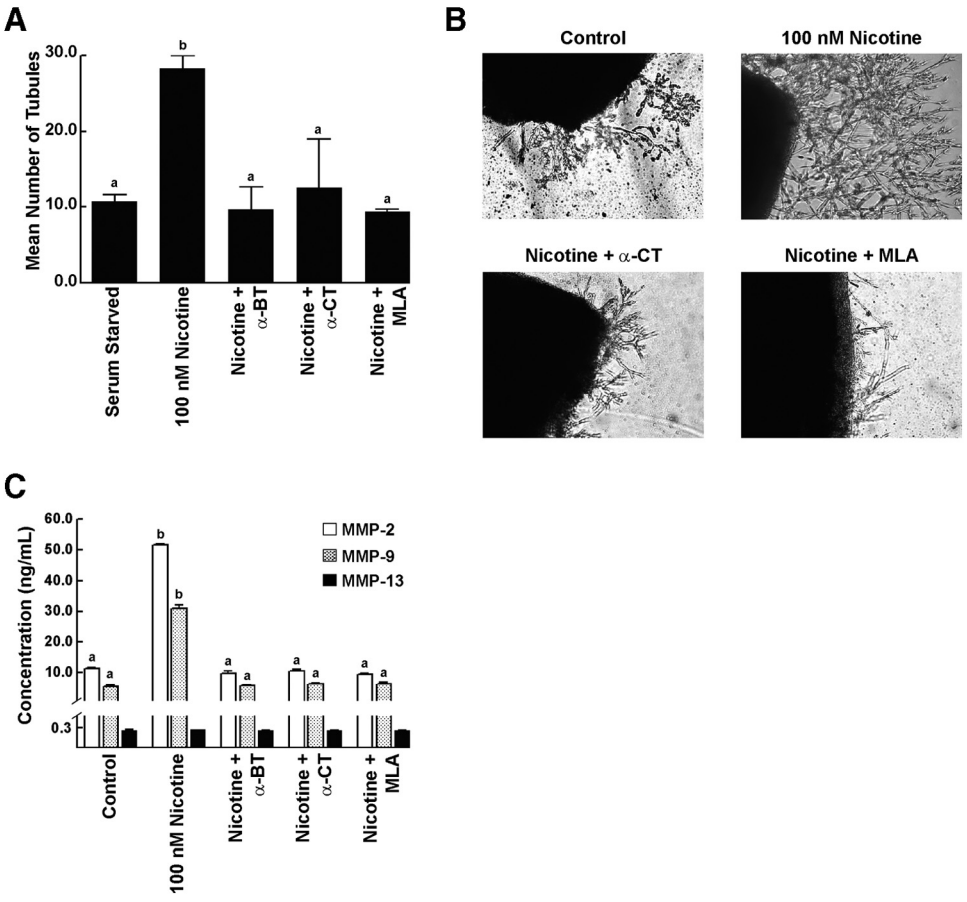

FIGURE 7. Antagonists to α7-nAChR abrogated the proangiogenic activity of nicotine in HRMECs. (**A**) Matrigel duplex assays demonstrated that the treatment of HRMECs with 1 μM of the α7-nAChR antagonists, α-BT, α-CT, or MLA, significantly suppressed the proangiogenic activity of nicotine. (**B**) Rat retinal explant assays showed that nicotine-induced angiogenic sprouting was abrogated by 1 μM of α-CT (*bottom left*) and 1 μM of MLA (*bottom right*). (**C**) ELISA assays indicated that treatment of HRMECs with 1 μM of the α7-nAChR antagonists, α-BT, α-CT, or MLA, reduced nicotine-induced secretion of MMP-2 and -9. MMP-13 levels were unaffected by nicotine and the α7-nAChR antagonists.

have been characterized by confocal laser microscopy and transmission electron microscopy.[35] Use of the Matrigel duplex assay means that the data obtained in the present study are more relevant to the angiogenic processes in the retina.[34,35]

The proangiogenic activity of nicotine was mediated by the upregulation of matrix metalloproteinases (MMPs) MMP-2 and -9 in HRMECs. MMPs, a family of approximately 25 members, are zinc enzymes that can degrade proteins of the extracellular matrix. They regulate major cellular functions like physiological angiogenesis, cellular movement and migration, tissue repair, and cell signaling.[41,63] We chose MMP-2 and -9 in the present study because they have been known to play a pivotal role in retinal angiogenesis.[51–53] MMP-2 and -9 are gelatinases that digest type IV collagen in the extracellular matrix.[64] Previous studies have demonstrated that the treatment of retinal endothelial cells and retinal pericytes with advanced glycation end products (produced during diabetes) upregulates the expression of MMP-2.[51] Similarly, MMP-9 has been shown to be upregulated in the retina of diabetic rats.[65] MMP-9 also acts as a chemoattractant, enabling the migration of inflammatory cells such as neutrophils and monocytes, which are important for endothelial cell migration during retinal neovascularization.[66] Clinical studies show that MMP-2 and -9 levels are activated in the retinal, vitreous and fibrovascular tissues in patients with diabetic retinopathy and ARMD.[45] In addition, growth factors like bFGF and VEGF upregulate the secretion of MMP-2 and -9 in retinal cells during angiogenesis.[55] Taken together, MMP-2 and -9 facilitate endothelial cell remodeling, disrupt tight-junctions and increase vascular permeability and perturbation of the blood–retinal barrier during diabetic retinopathy.[41,45–50]

Data presented in Figure 5A shows that nicotine-induced angiogenesis was mediated by upregulation of MMP-2 and -9 in HRMECs and in rat retinal explants. In contrast, Kiuchi et al.[25] found that MMP-2 and -9 were not involved in the proangiogenic activity of nicotine in the laser-induced CNV model in mice. This result may be explained by differences in cell types used in the experiment. Kiuchi et al. studied the effect of nicotine on MMP-2 and -9 levels in choroidal vascular smooth muscle cells of mice, whereas we explored the effect of nicotine on MMP levels in retinal endothelial cells. Furthermore, our experiments were performed using the Matrigel duplex and rat retinal explant assays, whereas Kiuchi et al. used the laser-induced CNV model in mice. We also studied the effect of nicotine on the levels of TIMP-1 and -2, which bind to MMP-2 and -9 and inhibit their gelatinase activity. We observed that the treatment of HRMECs with 100 nM nicotine caused significant ($P < 0.05$) suppression of both TIMP-1 and -2 levels. To our knowledge, this study is the first to report the effect of nicotine on expression of TIMPs in retinal endothelial cells. Our results agree with those of Katono et al.,[67] whose studies showed a decrease in the production of TIMPs (1, 3, and 4) in human osteoblasts on long-term exposure to nicotine.

Figure 8 is a highly simplified summary of how α7-nAChR-signaling may promote retinal angiogenesis. The activation of α7-nAChRs by nicotine stimulates the angiogenic switch in retinal endothelial cells by increasing production of MMP-2 and -9 and simultaneously suppressing the expression of inhibitory proteins such as TIMP-1 and -2. Previous studies have shown that nicotine can activate phospholipase A2 and increase the recruitment of endothelial progenitor cells in the vasculature of the retina.[32] However, the upstream signaling pathways that actually link the activation of α7-nAChRs to MMPs, TIMPs,

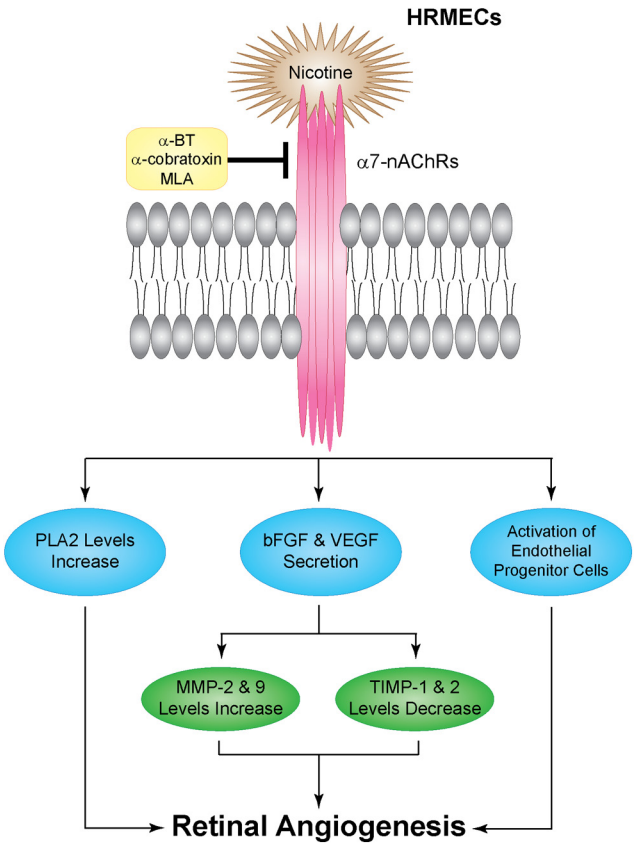

FIGURE 8. A highly simplified summary of the pathways underlying the proangiogenic activity of nicotine in HRMECs. Nicotine binds to high-affinity α7-nAChRs on HRMECs to stimulate the production of MMP-2 and -9 and inhibit levels of TIMP-1 and -2. Both of these processes eventually promote retinal angiogenesis. Another signaling pathway induced by nicotine in HRMECs involves activation of phospholipase A2. Nicotine can also increase the recruitment of endothelial progenitor cells to stimulate neovascularization in the retina. The pathways which connect α7-nAChRs to these downstream signaling proteins are unknown, but they probably involve the secretion of growth factors like bFGF or VEGF in response to nicotine treatment. Taken together, α7-nAChR antagonists could be useful for attenuating the proangiogenic activity of nicotine in retinal endothelial cells.

phospholipase A2 and subsequent angiogenesis are yet to be elucidated. Studies in angiogenic models of cancer suggest that nicotine can induce the secretion of growth factors like bFGF and VEGF, which activate downstream angiogenic pathways.[23,68–70] It is tempting to speculate that the same signaling networks are responsible for the proangiogenic effects of nicotine in the retina. Our future studies will address the upstream signaling mechanism by which the α7-nAChR links to the MMP pathway and promotes retinal angiogenesis.

Our data show that nAChR function is essential for the proangiogenic effect of nicotine in two independent assays of retinal angiogenesis. Our results concur with data reported by Kiuchi et al.[25] and Suner et al.,[24] showing that nicotine promoted angiogenesis in laser-induced CNV models in mice. They also found that the proangiogenic activity of nicotine was reversed by the administration of the generalized nAChR antagonists hexamethonium and mecamylamine. However, a potential disadvantage of generalized nAChR inhibitors is that they bind to all nAChR subunits and therefore increase the likelihood of unwanted side effects. These observations underscore the need for nAChR-subunit–specific inhibitors with high antiangiogenic activity.

To the best of our knowledge, there are no reports that identify the specific nAChR subunits responsible for the proangiogenic activity of nicotine in the retina. The present study fills this void in knowledge and shows for the first time that the α7-nAChR mediates the angiostimulatory effects of nicotine on retinal endothelial cells. Data from other experimental systems have indicated that α7-nAChRs primarily mediate endothelial cell proliferation, invasion, and angiogenesis in lung cancer.[27–29] The α7-nAChR is upregulated during hypoxia-induced proliferation of endothelial cells[26,60,62] and in murine models of atherosclerosis.[26,60] These findings emphasize the vital role played by α7-nAChR in regulating pathologic angiogenesis, activated by exogenous nicotine from cigarettes. In the present study, we demonstrated that α7-nAChR inhibitors significantly abrogate nicotine-induced angiogenesis in both the Matrigel duplex and rat retinal explant assays ($P < 0.05$). The antiangiogenic activity of these α7-nAChR inhibitors correlated with the suppression of MMP-2 and -9 levels in HRMECs. Taken together, our data suggest that the α7-nAChR may represent a novel molecular target for the treatment of neovascular diseases of the retina.

The primary objective of the present study was to provide the proof of principle of this concept. Our future studies will identify novel α7-nAChR antagonists by high-throughput screening of small molecule combinatorial libraries. The antiangiogenic activity of these top hit compounds will be determined in cell culture, Zucker diabetic fatty rat (ZDF/Gmi-Fa) models, and oxygen-induced retinopathy models.[71,72] Using this approach, we hope to identify novel α7-nAChR antagonists with high selectivity and potent antiangiogenic activity. We believe these studies pave the way for novel α7-nAChR-based treatment for retinopathy patients who are active smokers,[23,60] are exposed to second-hand smoke, or use nicotine patches or gums for smoking cessation.[30,73]

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